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XX. THE INHIBITION OF LACTIC ACID FORMATION IN CANCER AND MUSCLE.

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From a chemical standpoint, the problem of cancerous growth may be considered in two ways, either that disordered growth is caused by the presence of a chemical factor not present in normal tissue, or that disordered growth is caused by the absence of some factor normally present in adult tissue. The latter point of view seems, perhaps, to be the more plausible when one considers that, in certain respects, the metabolism of embryonic tissue and that of cancer tissue are similar [Warburg, 1924]. It seems reasonable to assume that with the development of the embryo there also develops a factor which checks certain phases of embryonic metabolism. This factor, present in normal adult tissue, would act as a growth regulator. If, in later life, the factor for any reason disappears, the tissue would again assume certain embryonic characteristics. In this paper experiments are described which were carried out to determine whether the addition of normal tissue extracts to cancer could change the characteristic metabolism of malignant tissue. As is well known, the work of Warburg [1923–1926] has shown that the most characteristic of the differences so far observed between the metabolism of cancerous tissue and that of normal tissue is the marked glycolytic power of the former. The line of experimentation followed, then, was to observe the change in glycolytic activity of cancer tissue brought about by the addition of extracts of various normal tissues.

Different regions of the body seem to possess a markedly different susceptibility to cancerous growths. The small intestine apparently affords a site very unfavourable to the growth of malignant tissue, since cancer in that region is extremely rare. On the line of thought suggested above, it might be supposed that the small intestine contained some chemical factor which checked cancerous growth, a factor either produced by the intestine itself or by one of those organs which empty their secretions into the small intestine. The pancreas thus suggested itself as the first tissue to use for our experimental purpose. A further reason for this choice was afforded by the observation of Winfield and Hopkins [1915], and later of Foster and Woodrow [1924] and Foster [1925], that extracts of pancreas had an inhibiting effect on lactic acid formation by muscle hash. McCullagh [1928] showed that pan-

creatic extracts also inhibit lactic acid formation in the soluble muscle enzyme system of Meyerhof [1926]. The present paper shows the effect of pancreatic extracts on cancer metabolism. This work was begun in May, 1928, and many of the experiments to be described had been completed when in December, 1928, a paper appeared by Barr, Ronzoni and Glaser [1928] in which the authors described experiments which had been undertaken with the same aim as our own, namely, to see whether pancreatic preparations inhibited the glycolysis of cancer tissue. Taking their results as a whole, they concluded that pancreatic extract did not inhibit this glycolysis. Our own experiments had led us to the opposite conclusion, and since some inhibition had been obtained in a number of Barr, Ronzoni and Glaser's experiments, we decided to continue our work.

Experimental.

Warburg has shown that not only is the anaerobic glycolysis of cancer tissue high, but also the aerobic glycolysis, whereas normal adult tissue shows practically no aerobic glycolysis. We therefore studied first the effect of pancreatic extracts on the aerobic glycolysis of cancer tissue. The type of manometric apparatus and technique described by Warburg were used. In all of these experiments, a thin slice of cancer tissue was suspended in bicarbonate Ringer solution (0·25 *M* with respect to bicarbonate). When glycolysis was being measured, the Ringer contained 0·2 % glucose. Each apparatus was filled with a mixture of 95 % air and 5 % CO_2 and shaken in a water-bath at 37° for 1 hour. At the end of the experiment, the tissue was removed, washed, dried and weighed. A watery extract of commercial pancreatin was made by allowing 0·5 g. of the dry preparation to stand in 5 cc. water for an hour and then filtering. This preparation was made fresh every day. Another extract was made according to the method of Foster and Woodrow [1924] in which the factor is precipitated by 70 % alcohol. In all the later experiments, the pancreatic preparation from fresh pancreas described by Ronzoni, Glaser and Barr [1928] was used. This is precipitated by 58 % alcohol. Preliminary experiments showed that heated pancreatic extract did not inhibit the glycolysis of cancer tissue. In investigating the action of the pancreatic extracts, the same amount of heated extract was always added to the control apparatus as of unheated extract to the experimental apparatus, the two solutions being in this way more comparable than if water were used in the control. The pancreatic extract for the control was immersed in a test-tube in boiling water for 5 minutes. The p_H of heated and unheated extracts was brought to 7·6 before using.

The malignant tissue used was for the most part mouse carcinoma 63, and occasionally the slow growing Twort carcinoma, both kindly supplied by Dr J. A. Murray, F.R.S., of the Imperial Cancer Research Institute. Rat carcinoma 9 was tried but great difficulty was experienced in getting tumours that were not necrotic or haemorrhagic.

A typical experiment using mouse carcinoma 63 is given below. Five Barcroft micro-apparatus, of the type having the manometer open at one end, were used, containing:

1. 0·4 cc. bicarbonate Ringer + 0·1 cc. pancreatic extract + 2·8 mg. tissue:
2. 0·4 cc. bicarbonate Ringer + 0·1 cc. heated pancreatic extract + 2·2 mg. tissue:
3. 0·4 cc. bicarbonate Ringer (0·2 % glucose) + 0·1 cc. pancreatic extract + 1·6 mg. tissue:
4. 0·4 cc. bicarbonate Ringer (0·2 % glucose) + 0·1 cc. heated pancreatic extract + 1·8 mg. tissue.

A fifth apparatus containing 0·5 cc. Ringer only was used as a temperature control. Apparatus 1 and 2 contained no glucose and therefore gave the CO_2 produced by oxidation processes. Apparatus 3 and 4 contained glucose, and any CO_2 given off beyond that produced in 1 and 2 was due to the glycolysis of glucose [Warburg, 1923]. The figures (multiplied by the apparatus constant and divided by tissue weight) for the above experiment are given below. They represent mm.^{3} CO_2 per mg. dry weight of tissue per hour.

1.	Ringer + pancreatic extract	+ 0·5
2.	,, + heated extract	− 1·7
3.	,, + glucose + pancreatic extract	+ 14·6
4.	,, + glucose + heated extract	+ 20·2

Glycolysis with pancreatic factor = (14·6 − 0·5) = 14·1
,, without ,, ,, = (20·2 + 1·7) = 21·9

In this experiment the pancreatic extract has caused an inhibition in aerobic glycolysis of 35 %. Previous workers using muscle hash had obtained inhibitions ranging from 40 to 60 %.

A possible source of error lay in the fact that the buffering power of the pancreatic extract might have been lowered by heating and that hence the heated extract would not retain so much CO_2 as the unheated. If this were true, a correction factor would have to be applied to the observed readings. To test this possibility, an experiment was carried out according to Negelein's [1925] method, and it was found that no extra retention of CO_2 occurred in the solution containing unheated pancreatic extract and that therefore no retention correction was necessary.

Table I gives the results of experiments measuring the aerobic glycolysis of cancer tissue as affected by extracts of pancreas. The pancreatic extract made according to Foster and Woodrow is designated as F and W. The extracts marked "sterilised" were passed through a sterilising filter.

It will be seen from Table I that in 15 of the 22 experiments the pancreatic extract caused an inhibition of glycolysis ranging from 15 to 100 %. During the course of these experiments, the work of Case and McCullagh [1928] appeared, which showed that the pancreatic factor inhibiting muscle glycolysis

Table I.

No. of exp.	Cancer tissue	Pancreatic extract cc.		mm.³ CO_2 per mg. per hour: Without glucose	With glucose	Aerobic glycolysis	Inhibition %
1	Twort	Water extract	0·01 heated	− 1·35	+15·18	16·5	
			0·01	+ 1·4	+ 6·9	5·5	66
2	63	Water extract	0·1 heated	− 0·6	+ 8·6	9·2	
			0·1	+ 7·8	+12·0	4·4	52
3	63	Water extract	0·1 heated	+ 1·4	+19·2	18·8	
			0·1	+20·1	+30·8	12·7	32
4	63	Water extract	0·1 heated	+ 0·5	+15·7	15·2	
			0·1	+ 8·1	+ 8·1	0·0	100
5	63	Water extract	0·05 heated	+ 0·2	+19·1	18·9	
			0·05	+ 6·0	+21·9	15·9	16
6	63	F and W	0·1 heated	− 2·9	+ 4·9	7·8	
			0·1	+ 2·8	+ 4·7	1·9	75
7	63	F and W	0·1 heated	+ 3·5	+16·9	13·4	
			0·1	+ 6·7	+ 8·0	1·3	90
8	Spontaneous mouse tumour	Water extract	0·05 heated	− 0·5	+21·6	22·1	
			0·05	− 1·0	+16·9	17·9	19
9	63	Water extract sterilised	0·05 heated	− 2·8	+10·9	13·7	
			0·05	+ 2·4	+11·1	8·7	36
10	Twort	Water extract	0·05 heated	− 3·6	+11·6	15·2	
			0·05	+33·0	+27·4	—	100
11	63	Water extract	0·05 heated	− 0·4	+19·3	19·7	
			0·05	+29·0	+36·0	7·0	64
12	63	Water extract sterilised	0·05 heated	+ 2·4	+ 7·9	5·5	
			0·05	+12·4	+14·1	1·7	69
13	63	Water extract	0·05 heated	0·0	+14·9	14·9	
			0·05	+ 8·6	+14·1	5·5	63
14	63	Water extract	0·02 heated	+ 1·3	+15·8	14·5	No inhibition
			0·02	+ 2·9	+18·0	15·1	
15	Twort	Water extract	0·05 heated	+ 0·7	+ 9·3	8·6	
			0·05	+ 3·7	+10·8	7·1	17
16	63	Water extract	0·05 heated	− 0·9	+ 6·2	7·1	No inhibition
			0·05	+ 8·0	+24·2	16·2	
17	63	Water extract sterilised	0·05 heated	− 1·8	+ 3·7	5·5	No inhibition
			0·05	+ 6·3	+16·2	9·9	
18	63	Water extract	0·05 heated	+ 2·8	+ 8·0	5·2	
			0·05	+ 3·2	+ 7·6	4·4	15
19	63	Water extract	0·05 heated	+ 1·1	+ 4·1	3·0	No inhibition
			0·05	+ 4·8	+ 7·9	3·1	
20	63	F and W No amylase	0·05 heated	+ 8·2	+15·3	7·1	No inhibition
			0·05	+ 5·2	+20·6	15·0	
21	63	F and W No amylase	0·1 heated	− 1·6	+ 7·9	9·5	No inhibition
			0·1	+ 0·2	+13·8	13·6	
22	63	F and W No amylase	0·1 heated	− 0·8	+ 6·8	7·6	No inhibition
			0·1	+ 2·0	+11·6	9·6	

of starch was in all probability amylase. All extracts after that were tested for amylase. In three of the seven experiments (Nos. 20, 21 and 22) in which the pancreatic extract did not cause an inhibition of glycolysis, alcoholic extracts made according to Foster and Woodrow's directions were used. Each of these extracts was tested for amylase and gave a practically negative result. In another of the experiments (19) in which there was no inhibition, the aerobic glycolysis without pancreas was already very low, 3 mm.³ CO_2 being the production by 1 mg. of tissue in 1 hour as compared with an average

of 12 mm.^{3} per hour. Discarding those experiments in which the alcoholic extracts contained no amylase, the results in Table I show that pancreatic extracts caused an inhibition in the aerobic glycolysis of cancer tissue in 15 out of 19 cases. The solution containing unheated pancreatic extract and no glucose generally gave off more CO_2 than did that containing heated extract. It is unfortunate that the smaller figure for glycolysis with pancreatic extract is obtained in some cases because the larger control is subtracted, but this does not apply to the malt diastase experiments nor to the anaerobic and chemical experiments to be given later which show that the lower glycolysis with unheated pancreatic extract is real.

Table II.

No. of exp.	Carcinoma	Inhibitor cc.		mm.^{3} CO_2 per mg. per hour: Without glucose	With glucose	Aerobic glycolysis	Inhibition %
1	63	Malt diastase	0·05 heated	+ 2·4	+16·9	14·5	
			0·05	+ 2·8	+ 6·9	4·1	71
2	63	Malt diastase	0·1 heated	+ 3·3	+21·7	18·4	
			0·1	+ 5·6	+11·7	6·1	66
3	63	Malt diastase	0·1 heated	+ 4·8	+11·9	7·1	
			0·1	+ 3·8	+ 5·6	1·8	74
4	63	Malt diastase	0·1 heated	+ 4·0	+11·1	7·1	
			0·1	+ 3·8	+ 9·4	5·6	21
5	63	Malt diastase	0·05 heated	+ 0·3	+33·8	33·5	
			0·05	+ 4·8	+16·0	11·2	63
6	63	Malt diastase sterilised	0·05 heated	+ 2·5	+ 5·6	3·1	
			0·05	+ 2·8	+ 4·7	1·9	39
7	63	Malt diastase sterilised	0·1 heated	− 1·4	+11·3	12·7	
			0·1	+ 2·4	+ 5·6	3·2	74
8	63	Malt diastase sterilised	0·1 heated	+ 0·4	+ 9·4	9·0	
			0·1	+ 3·4	+ 8·9	5·5	39
9	63	Malt diastase half strength	0·05 heated	− 1·9	+ 3·0	4·9	No inhibition
			0·05	− 1·2	+ 4·6	5·8	
10	Twort	Takadiastase	0·1 heated	+ 3·2	+ 9·7	6·5	
			0·1	+ 8·6	+ 9·2	0·6	90
11	Twort	Takadiastase	0·1 heated	+ 1·9	+22·7	20·8	
			0·1	+14·0	+10·2	—	100
12	Twort	Takadiastase	0·1 heated	0·0	+10·4	10·4	
			0·1	+ 7·3	+12·4	5·1	51
13	63	Takadiastase	0·1 heated	+ 1·5	+ 7·6	6·1	
			0·1	+14·0	+13·5	—	100
14	63	Takadiastase	0·1 heated	+ 0·7	+ 6·8	6·1	
			0·1	+ 6·1	+ 8·0	1·9	69
15	63	Takadiastase	0·1 heated	+ 1·0	+12·3	11·3	
			0·1	+ 3·9	+11·9	8·0	29
16	63	Takadiastase	0·1 heated	+ 3·7	+ 8·1	4·4	
			0·1	+ 7·5	+10·9	3·4	22
17	63	Takadiastase	0·1 heated	+ 6·8	+13·1	6·3	No inhibition
			0·1	+ 5·5	+14·4	8·9	
18	63	Salivary gland	0·1 heated	+ 6·0	+23·4	17·4	
			0·1	+ 5·4	+12·2	6·8	61
19	63	Salivary gland	0·1 heated	+ 2·6	+ 9·2	6·6	
			0·1	+ 2·8	+ 7·2	4·4	33
20	63	Salivary gland	0·1 heated	+ 2·2	+10·4	8·2	
			0·1	+ 4·4	+ 9·4	5·0	39
21	63	Salivary gland	0·1 heated	+ 0·8	+ 7·9	7·1	
			0·1	+ 3·2	+ 9·6	6·4	9
22	63	Salivary gland	0·1 heated	+ 0·2	+ 4·7	4·5	No inhibition
			0·1	0·0	+ 5·0	5·0	

Since Case and McCullagh had shown the inhibiting factor for muscle glycolysis to be present in other extracts containing amylase, we tried the effect of such extracts on the aerobic glycolysis of cancer tissue. Malt diastase and takadiastase extracts were made by extracting 0·5 g. dried commercial preparation with 5 cc. water for 1 hour, filtering and adjusting to about p_H 7·6. The salivary gland preparation was made as follows. The submaxillary gland of an ox was minced, ground with acetone twice, filtered, ground with ether twice, filtered and dried in air. 0·2 g. was extracted in 8 cc. water.

The effects of malt diastase, takadiastase and salivary gland extracts on the aerobic glycolysis of cancer tissue are shown in Table II.

Table II shows that in 22 experiments in which malt diastase, takadiastase or dried salivary gland was used, there were 19 cases in which the presence of one or other of these preparations caused an inhibition in the aerobic glycolysis of cancer tissue.

Similar experiments using pancreatic extracts were then made anaerobically. The experiments were carried out in the manner already described except that the gas mixture used for filling the flasks consisted of 95 % nitrogen and 5 % CO_2. The results are given in Table III. The pancreatic extract used in these experiments was prepared according to the method of Ronzoni, Glaser and Barr.

The tissue used in all the experiments in this table was mouse carcinoma 63.

Table III.

No. of exp.	Pancreatic extract cc.	mm.^{3} CO_2 with glucose	Inhibition %
1	0·1 heated	29·5	
	0·1	21·1	28
2	0·1 heated	11·1	
	0·1	1·2	89
3	0·1 heated	15·8	
	0·1	3·3	79
4	0·1 heated	6·5	
	0·1	1·0	84
5	0·1 heated	24·0	
	0·1	13·0	46
6	0·1 heated	10·2	
	0·1	7·5	26
7	0·1 heated	11·7	
	0·1	0·4	96

It can be seen from Table III that pancreatic extract made according to Ronzoni, Glaser and Barr's directions caused an inhibition (ranging from 26 to 96 %) in the anaerobic glycolysis of cancer tissue in all of the seven experiments.

Several experiments were then carried out on a larger scale, in which the lactic acid formed was estimated directly by the chemical method of Friedemann, Cotonio and Shaffer [1927]. The pancreatic extracts for these experiments were made according to Ronzoni, Glaser and Barr, a number of different preparations being used. A control was always done using Ringer

solution and pancreatic extract without tissue. The iodine value for this was subtracted from that obtained when a similar solution contained tissue. Also a control experiment was always made with tissue in Ringer with no added sugar to allow for the lactic acid from the tissue alone. The reading obtained from this control was subtracted from that obtained for the sugar-containing solutions both with and without pancreatic extract. 14 cc. bicarbonate Ringer, 5 cc. pancreatic extract, or 5 cc. water (or in some cases 5 cc. heated extract) and 1 cc. glucose solution (of such strength that the final glucose concentration was 0·4 %) were put into a flask and a weighed amount of cancer tissue slices added. (A number of tumours were sliced thinly, the slices drained quickly on filter paper, and, having been picked at random from the mixed tumours, were weighed, about 0·4 to 0·5 g. being added to each flask.) The flasks were filled with nitrogen containing 5 % CO_2, stoppered and shaken in a water-bath at 37° for 2 hours. Before the estimation of lactic acid, proteins were removed by trichloroacetic acid and sugars by copper sulphate and calcium hydroxide. Table IV gives the results of these experiments. P.E. designates pancreatic extract.

Table IV.

No. of exp.	Cancer tissue	Mg. lactic acid formed per g. dry wt. tissue		Inhibition %
1	63	No P.E.	31·5	
		With P.E.	12·0	61
2	Human fibro-sarcoma of leg	No P.E.	32·8	
		With P.E.	0·0	100
3	63	No P.E.	16·5	
		With P.E.	9·5	42
4	63	No P.E.	48·7	
		With P.E.	22·7	53
5	63	No P.E.	18·0	
		With P.E.	0·2	99
6	63	No P.E.	27·5	
		With P.E.	22·0	20
7	63	No P.E.	46·5	
		With P.E.	19·0	59
8	63	No P.E.	37·0	
		With P.E.	30·0	19
9	63	No P.E.	48·0	
		With P.E.	6·0	87
10	63	No P.E.	61·0	
		With P.E.	20·0	67
11	63	No P.E.	80·5	
		With P.E.	26·0	67
12	63	No P.E.	37·5	
		With P.E.	7·2	80
13	63	No P.E.	30·0	
		With P.E.	3·0	90
14	63 + fructose	No P.E.	30·4	
		With P.E.	6·9	77
15	63 + fructose	No P.E.	22·5	
		With P.E.	6·5	71

Table IV shows that in the 13 experiments in which glucose was used the pancreatic extract in all cases caused an inhibition in the amount of lactic

acid formed, ranging from 20 to 100 %. Warburg has shown that cancer tissue can use fructose also as a precursor of lactic acid. In the last two experiments in Table IV, 0·4 % fructose was used instead of glucose and it can be seen that pancreatic extract inhibits this glycolysis also.

Barr, Ronzoni and Glaser [1928] quote a total of 21 manometric experiments on the glycolysis of cancer tissue in the presence of pancreatic extract, 8 cases of inhibition occurring, ranging from 6 to 37 %. They also give 6 chemical experiments in 2 of which inhibition occurred, to the extent of 5 and 29 % respectively. They conclude that pancreatic extract causes no appreciable inhibition in the glycolysis of cancer tissue. If all the experiments in the previous four tables are considered together (not counting the 3 in which the pancreatic extract had no amylase), there is a total of 63, with only 4 where no inhibition occurred. We feel justified, then, in stating that some factor, presumably amylase, common to pancreatic extract, malt diastase, takadiastase and dry salivary gland, does cause an inhibition in the glycolysis of the cancer tissue used in this laboratory.

The question now arises as to the mechanism by which these various extracts bring about inhibition. McCullagh has shown that the substance which brings about the inhibition in muscle glycolysis when starch is used is amylase. After the publication of this work, tests for amylase were always made on the various extracts used in our work, and we found that extracts containing no amylase gave no inhibition in the glycolysis of cancer tissue. It is difficult to make any accurate comparison using cancer tissue between the degree of inhibition caused by various preparations and their amylase content, because the use of slices of tissue, rather than the homogeneous tissue extract which McCullagh used from muscle, involves an experimental error which invalidates any but fairly large differences. Barr, Ronzoni and Glaser made an extract of cancer tissue but found it to be glycolytically inactive. It is therefore, at the moment, impossible to work with a more homogeneous source of the glycolytic enzyme from cancer tissue than is provided by slices of tissue. We assume, then, for the time being, that the factor inhibiting glycolysis in cancer tissue is amylase. McCullagh claims that amylase inhibits the glycolysis of starch because it prevents the formation of hexosephosphate which is known to be an intermediate product in the breakdown by muscle of starch to lactic acid. Barr, Ronzoni and Glaser, who added phosphate to bicarbonate-Ringer, observed no increase in the lactic acid formation by cancer tissue, and hence they assumed that glucose, when acted upon by cancer tissue, did not go through a phosphate stage. This assumption was supported by their inability to observe any changes in inorganic phosphorus during the course of glycolysis in cancer tissue. They therefore explain their observations that in the majority of cases pancreatic extract did not inhibit the glycolysis of cancer tissue, by agreeing with McCullagh as to the function of the inhibiting factor, namely that in muscle it prevents the formation of hexosephosphate. If, in cancer meta-

bolism, hexosephosphate plays no part, on the above assumption no inhibition of glucose glycolysis would be expected. But, as the previous tables have shown, in 95 % of our experiments pancreatic extract (or some other amylase-containing extract) did cause an inhibition of glycolysis in cancer tissue. We then made a number of experiments, using hexosediphosphate and hexose-monophosphate as substrates for glycolysis instead of glucose. Barium hexosediphosphate and barium hexosemonophosphate were very kindly given to us by Dr R. Robison, who informed us that the hexosemonophosphate was of the usual mixed aldose-ketose type, obtained by fermentation with yeast juice and purified by precipitation as basic lead salt.

We also wish to express our thanks to Bayer Products, Ltd., for very kindly presenting us with a quantity of calcium hexosediphosphate. The hexose-phosphate solutions were made up so that they were equivalent to a final concentration of 0·4 % hexose, thus making them comparable with solutions containing 0·4 % glucose. Calcium hexosediphosphate was dissolved in the molecular equivalent amount of sodium oxalate, and the calcium oxalate centrifuged off, the supernatant liquid always being tested to ensure freedom from oxalate. The analysis provided by Bayer Products, Ltd. of the calcium hexosediphosphate showed that it contained 29 % water, an allowance for which was made in the quantities used. Barium hexosemonophosphate was treated with the molecular equivalent amount of Na_2SO_4, centrifuged and tested for freedom from Ba. The hexosediphosphate solution was prepared in the same way. Several experiments were done anaerobically using the manometric method already described. The results of these experiments are given in Table V. Carcinoma 63 was used. P.E. represents pancreatic extract.

Table V.

No. of exp.	Substrate	mm.^{3} CO_2 per mg. dry wt. tissue per hour
1	Glucose	29·5
	„ +P.E.	21·1
	Hexosediphosphate	3·9
	„ +P.E.	0·8
2	Glucose	11·1
	„ +P.E.	1·2
	Hexosediphosphate	2·6
	„ +P.E.	4·4
3	Glucose	15·8
	„ +P.E.	3·3
	Hexosediphosphate	0·0
	„ +P.E.	0·0
4	Glucose	6·5
	Hexosediphosphate	0·0
5	Glucose	10·2
	Hexosediphosphate	0·0
	Hexosemonophosphate	0·0
6	Glucose	11·7
	„ +P.E.	0·4
	Hexosemonophosphate	0·8
	„ +P.E.	1·5

Several experiments, in which the lactic acid was determined chemically, were carried out using these substrates. Their results are given in Table VI. Carcinoma 63 was used.

Table VI.

No. of exp.	Substrate	Mg. lactic acid per g. dry wt. tissue
1	Glucose	55·0
	Hexosediphosphate	0·0
2	Glucose	30·0
	„ +P.E.	3·0
	Hexosediphosphate	1·7
	„ +P.E.	0·0
	Hexosemonophosphate	0·0
	„ +P.E.	0·0
3	Fructose	22·5
	„ +P.E.	6·5
	Hexosediphosphate	7·0
	„ +P.E.	0·0
	Hexosemonophosphate	2·5

The experiments in Tables V and VI show that cancer tissue cannot use either hexosediphosphate or hexosemonophosphate to any appreciable extent as a source of lactic acid. Starch was tried also as a substrate for cancer glycolysis but the tumour tissue was unable to act upon it, as Barr, Ronzoni and Glaser have also found. It is clear that the hypothesis that pancreatic extract inhibits glycolysis by preventing the formation of hexosephosphate is not applicable at any rate to cancer tissue, since the latter cannot form lactic acid from either the diphosphate or the monophosphate when they are supplied.

The possibility that the inhibition might be a simple adsorption of glucose by the unheated extract is rendered unlikely by the result of the following experiment in which doubling the concentration of glucose, leading to an increase of free sugar in the solution, produced no appreciable change in the inhibition brought about by a given amount of pancreatic extract. Carcinoma 63 was used.

Substrate	mm.^{3} CO_2 per mg. dry wt. tissue per hour	Inhibition %
Glucose 0·4 %	34·0	
„ „ +P.E.	13·0	46
Glucose 0·8 %	25·6	
„ „ +P.E.	14·6	43

As yet, then, we have no explanation to offer for the mechanism by which pancreatic extract inhibits the lactic acid formation in cancer tissue.

As a result of this work we decided to investigate McCullagh's theory of the cause of inhibition by amylase of glycolysis in muscle, according to which pancreatic extract inhibits the formation of lactic acid by preventing the intermediate formation of hexosephosphate. We have already shown that this theory does not explain the inhibition produced by pancreatic extract on the glycolysis in cancer tissue. We now tried the effect of pancreatic extract

on the soluble muscle enzyme system of Meyerhof [1926], to which hexosephosphate had already been added. The results are given in Table VII. For these experiments, the soluble muscle enzyme was prepared according to the directions of McCullagh. The quantities used in each flask were 8 cc. of muscle extract, 3 cc. phosphate buffer (made according to McCullagh), 4 cc. pancreatic extract (Ronzoni, Glaser and Barr's), 3 cc. 2·4 % substrate (starch, glucose, or hexosephosphate which was 2·4 % with respect to hexose), and 2 cc. water or yeast activator (made according to Meyerhof [1927]). 5 cc. of the contents of each flask were removed immediately into 1 cc. of 20 % trichloroacetic acid, these solutions serving as controls. The remainder was incubated for 4 hours at 37°, and then 3 cc. of 20 % trichloroacetic acid were added. After filtering off the proteins, the sugars, starch and hexosephosphate were precipitated in an aliquot neutralised portion by $CuSO_4$ and $Ca(OH)_2$. This method precipitates not only glucose, but, as was shown by Embden and Zimmermann [1924], other carbohydrates including hexosephosphates. The lactic acid in an aliquot portion of the final filtrate was estimated by the method of Friedemann, Cotonio and Shaffer. Results are expressed in mg. of lactic acid produced by 10 cc. of soluble muscle enzyme. Controls were always done in which no substrate was added as there proved to be always a small formation of lactic acid with no added substrate. These control figures were subtracted from those obtained with solutions to which substrate was added. In Exp. 1, Table VII, barium hexosediphosphate was used. In the other experiments in this table, the calcium salt was used. P.E. designates pancreatic extract.

For Exp. 12 in Table VII, the water extracts of pancreas, takadiastase and malt diastase were made by extracting 2 g. of dry preparation with 30 cc. water for 2 hours, filtering and neutralising. The saliva was diluted 1 : 5.

In some of the experiments in which glucose was used as a substrate, an activator prepared from yeast by the method of Meyerhof was added. Meyerhof has shown that the soluble muscle enzyme system has in large part lost the power of changing glucose to lactic acid. This power he shows to be restored when yeast activator is added.

The effect of pancreatic extract on glycolysis in the soluble muscle enzyme system is seen from the results in Table VII to be as follows. (1) When starch is the substrate, inhibition is brought about; (2) when hexosediphosphate is the substrate, inhibition is again brought about; but (3) when hexosemonophosphate or (4) glucose plus yeast activator are the substrates, no appreciable inhibition occurs.

The fact that pancreatic extract inhibits glycolysis in the soluble muscle enzyme system not only when starch is the substrate but also when hexosediphosphate is used, shows that the inhibiting factor does not act by preventing the formation of hexosediphosphate, because when hexosediphosphate is added to the system, inhibition still occurs. The weight of evidence seems

Table VII.

No. of exp.	Substrate	Mg. lactic acid per 10 cc. muscle extract	Inhibition %
1	Starch	27·3	
	,, +P.E.	22·6	17
	Glucose	4·9	
	,, +P.E.	0·0	100
	Hexosediphosphate	16·6	
	,, +P.E.	3·7	77
2	Starch	11·5	
	,, +P.E.	8·2	28
	Glucose	2·5	
	,, +P.E.	0·0	100
	Hexosediphosphate	9·0	
	,, +P.E.	0·0	100
3	Starch	16·0	
	,, +P.E.	0·6	91
	Glucose	3·1	
	,, +yeast activator	7·2	
	,, +yeast activator +P.E.	7·2	0
	Hexosediphosphate	8·9	
	,, +P.E.	3·5	60
4	Starch	7·2	
	,, +P.E.	0·9	87
	Hexosediphosphate	24·8	
	,, +P.E.	13·9	44
5	Starch	4·3	
	,, +P.E.	0·0	100
	Glucose	0·7	
	,, +P.E.	0·0	
	,, +yeast activator	3·3	
	,, +yeast activator +P.E.	5·0	0
	Hexosediphosphate	5·2	
	,, +P.E.	0·0	100
6	Starch	11·3	
	,, +P.E.	2·1	81
	Hexosediphosphate	17·8	
	,, +P.E.	8·8	50
	Hexosemonophosphate	20·5	
	,, +P.E.	19·2	6
7	Hexosemonophosphate	17·6	
	,, +P.E.	19·3	0
8	Starch	8·1	
	,, +P.E.	1·8	77
	Hexosediphosphate	18·9	
	,, +P.E.	9·0	52
	Hexosemonophosphate	13·0	
	,, +P.E.	13·5	0
9	Starch	15·6	
	,, +P.E.	8·6	45
	Hexosediphosphate	17·6	
	,, +P.E.	10·7	39
	Hexosemonophosphate	23·0	
	,, +P.E.	22·7	1
10	Starch	2·7	
	,, +P.E.	0·7	74
	Glucose +yeast activator	8·8	
	,, +yeast activator +P.E.	9·7	0
	Hexosediphosphate	6·0	
	,, +P.E.	1·8	70
	Hexosemonophosphate	17·3	
	,, +P.E.	14·9	13

Table VII (*cont.*)

No. of exp.	Substrate	Mg. lactic acid per 10 cc. muscle extract	Inhibition %
11	Starch	16·6	
	,, +P.E.	7·0	58
	,, +yeast activator	12·4	
	,, +yeast activator +P.E.	7·6	38
	Hexosediphosphate	6·4	
	,, +P.E.	1·0	84
	,, +yeast activator	11·0	
	,, +yeast activator +P.E.	0·0	100
	Hexosemonophosphate	13·3	
	,, +P.E.	15·8	0
12	Starch	14·9	
	,, +P.E. (R.G. and B.)	0·0	100
	,, +P.E. (water)	0·0	100
	,, +takadiastase	2·3	84
	,, +malt diastase	0·0	100
	,, +saliva	11·9	20
	Hexosediphosphate	18·9	
	,, +P.E. (R.G. and B.)	5·3	72
	,, +P.E. (water)	0·0	100
	,, +takadiastase	1·3	93
	,, +malt diastase	2·5	86
	,, +saliva	5·4	71

now, however, to point to hexosemonophosphate as being the normal precursor of lactic acid in muscle [Embden and Zimmermann, 1927; Meyerhof and Lohmann, 1927; Pryde and Waters, 1927]. Table VII shows that when hexosemonophosphate is added to the soluble muscle enzyme system, pancreatic extract no longer causes an inhibition in the formation of lactic acid. This observation is compatible with McCullagh's theory that the inhibiting power of the pancreatic extract is due to the prevention of esterification. When a substrate like glycogen is used from which hexosephosphate must be synthesised, the pancreatic extract causes inhibition in the amount of lactic acid formed, but when monophosphate itself is used, in which case obviously no synthesis is required, pancreatic extract causes no inhibition in lactic acid formation. Case and McCullagh showed that when glucose with yeast activator was added to the muscle enzyme, pancreatic extract caused no inhibition in lactic acid formation. Our own results (Table VII) confirm this observation. Meyerhof has shown that glucose with yeast activator and the muscle enzyme forms in part a labile hexosephosphate which is broken down to lactic acid. In other words, an esterification of glucose and phosphate must take place before lactic acid is formed. Case and McCullagh believe esterification to be impossible in the presence of pancreatic extract, yet their results and our own show that pancreatic extract in no way inhibits the lactic acid formation (and hence, according to Meyerhof, the hexosephosphate formation) from glucose acted upon by the muscle enzyme in the presence of yeast activator. Case and McCullagh state that since the inhibiting factor is amylase, and amylase does not act on glucose, no inhibition in lactic acid formation from glucose was to be expected. This deduction is difficult to

understand, because the function they have attributed to amylase, namely, the inhibition of esterification, is not one which it could exert on starch (its known substrate) since starch does not combine directly with phosphorus to make hexosephosphate. Meyerhof pictures that in the breakdown of starch there is first formed a labile hexose which then becomes esterified. It seems unlikely that the inhibiting factor would prevent esterification of this labile hexose from starch when it does not prevent esterification of glucose in the presence of its activator. It might appear at first sight possible that the yeast activator itself prevented the inhibiting action of pancreatic extract, but that such is not the case is clear from the results of Exp. 11, Table VII, where the addition of yeast activator to a muscle enzyme system acting on starch in the presence of pancreatic extract did not prevent the usual inhibition by the pancreatic extract from taking place. In this experiment, yeast activator was also added to the muscle enzyme system in which hexosediphosphate was the substrate. The inhibition produced by the pancreatic extract was not diminished by the presence of the activator. These observations led us to think that the inhibiting factor in the various extracts studied, while presumably amylase as Case and McCullagh believe, was not causing inhibition in lactic acid formation in muscle in the way they suggest, namely, by preventing esterification. McCullagh's evidence for his theory that hexosephosphate cannot be formed in the presence of amylase is, "When the formation of lactic acid was prevented by the use of the pancreatic factor, the total reducing power after acid hydrolysis sufficient to break down starch but not hexosephosphate was not changed. The hexosephosphates differ from glucose in their reducing power....These results, therefore, suggested that hexosephosphates were not being formed in the presence of pancreatic extract." Ronzoni, Glaser and Barr show a large increase in the amount of free reducing substances in the presence of the pancreatic factor. This increase in free reducing sugars is much greater in the presence of pancreatic extract than in its absence. These two facts considered together, first, that there is no loss of total carbohydrate and second, that there is a very large increase in free sugar in the presence of the pancreatic extract, might, from one point of view, be considered as evidence that, after the breakdown of glycogen to free sugar, the pancreatic factor exerts its activity, preventing this free sugar from becoming esterified. Hence the free sugar accumulates and the total carbohydrate remains the same. These observations, however, can be equally well explained on the hypothesis suggested below. The second piece of evidence advanced by Case and McCullagh that the pancreatic factor prevents esterification is the fact that in the presence of the pancreatic factor, inorganic phosphorus does not disappear, but in fact increases, whereas in a muscle enzyme system free from pancreatic extract, inorganic phosphorus decreases, pointing to the formation of a hexosephosphate. We consider that this evidence does not prove that the inhibiting power of pancreatic extract is due to the prevention of esterification of hexose and phosphate. We suggest that

a simpler explanation of the experimental observations can be based on the well-known fact that amylase breaks down starch to maltose. Meyerhof has shown that maltose yields only a very small amount of lactic acid when acted upon by the muscle enzyme system. Hence, the effect to be expected on adding pancreatic extract to a muscle enzyme system acting on starch would be a decreased lactic acid formation. Embden and Haymann [1924] have shown that maltose when added to muscle does not increase the formation of hexosephosphate; in other words, maltose will not link up with inorganic phosphorus in the presence of the muscle enzyme to form a hexosephosphate. This means, then, that in the presence of the pancreatic extract there would be no appreciable change in total free carbohydrates because no appreciable amounts of lactic acid or of hexosephosphate were being formed. But there would be a large increase in free sugar because starch was being broken down to maltose. In the uninhibited muscle system, starch is broken down to a labile hexose which is quickly esterified so that there is little increase in free sugar. Again, let us examine the inorganic phosphorus results of Case and McCullagh and Ronzoni, Glaser and Barr. In the uninhibited muscle system the amount of inorganic phosphate is reduced because hexosephosphate is built up more quickly than it is broken down. In the presence of pancreatic extract, inorganic phosphate does not disappear because maltose, which the amylase has formed from starch, does not link up with phosphorus to form hexosephosphate. In this manner, the results obtained by Case and McCullagh, Ronzoni, Glaser and Barr, and ourselves can be explained without assuming any unknown action of amylase on starch. Also this explanation, unlike that of McCullagh, is compatible with the results obtained with glucose and yeast activator.

Our results have shown that the production of lactic acid from hexosediphosphate is also inhibited by the pancreatic factor. Is this inhibition, like that of the starch, also due to amylase? Exp. 12, Table VII, shows the results of an attempt to find out whether the inhibition of lactic acid formation from these two substances was caused by the same factor. Several different amylase preparations were used and their effects on the glycolysis of starch and hexosediphosphate are in the main roughly parallel, so that, unless another factor were present in the various extracts in about the same proportion as amylase, the inhibition of the glycolysis of hexosediphosphate appears to be due to the same factor that causes the inhibition of the glycolysis of starch.

At present we cannot explain the mechanism by which pancreatic extract causes an inhibition in lactic acid formation from hexosediphosphate.

Several of Ronzoni, Glaser and Barr's experimental facts can be interpreted from our own results. They found, as did Foster and Woodrow, that with muscle itself, the pancreatic extract caused only 40–60 % inhibition. Ronzoni, Glaser and Barr show that this cannot wholly be explained by insufficient permeation of the muscle by the pancreatic extract and assume

that the incomplete inhibition is due to the presence of some preformed precursor of lactic acid. The precursor of lactic acid in muscle has been shown by various workers already quoted to be hexosemonophosphate. Our results show that hexosemonophosphate is not inhibited by the pancreatic extract and hence the incomplete inhibition obtained when pancreatic extract acted on muscle can be explained. Again, Ronzoni, Glaser and Barr give experiments in which the muscle enzyme system was for a time allowed to form hexosephosphate and then the inhibiting factor was added. After incubation, there was found to be some lactic acid formed in spite of the presence of the inhibitor. They explain that this is from the hexosephosphate formed before the inhibitor was added. This again fits in with our results in which the glycolysis of hexosemonophosphate is not inhibited by pancreatic extract. We suggest that what happens in the muscle enzyme system under various conditions is as follows:

Starch → Hexosemonophosphate → Lactic acid
↓ Pancreatic factor inhibits by forming
Maltose

Hexosediphosphate → Lactic acid
↓ Pancreatic extract inhibits possibly by forming
Free phosphate and a hexose not acted upon by muscle

Hexosemonophosphate → Lactic acid
↓ Pancreatic extract has no effect

Glucose + yeast activator → Hexosemonophosphate → Lactic acid
↓ Pancreatic extract has no effect

It is impossible at the moment to fit the cancer system into this scheme, as, in cancer, glucose glycolysis is inhibited by the pancreatic factor; also in muscle, glucose goes through a phosphate stage and in cancer it probably does not. It must be borne in mind, however, that in the muscle experiments an extract is used, while in the cancer experiments a slice of tissue is used. We are, however, able to say that in glycolysis by cancer tissue the pancreatic factor does not inhibit the formation of lactic acid by preventing the formation of hexosephosphates since the cancer tissue is unable to utilise such compounds when added.

The results obtained with cancer tissue indicate that the processes leading to the formation of lactic acid by cancer tissue are less complex than those in muscle. Muscle can form lactic acid not only from glucose but also from starch, hexosediphosphate and hexosemonophosphate, whereas cancer can use none of these substances except glucose as a source of lactic acid. Meyerhof has shown that the different stages of glycolysis in muscle are brought about by different enzymes. Our results indicate that cancer tissue contains an enzyme for glycolysing glucose but no enzymes for glycolysing starch and the hexosephosphates. The mechanism in cancer tissue by which glucose is changed to lactic acid is, according to our results, different from that in muscle. In the latter, glucose goes through a hexosephosphate stage before lactic acid is formed, whereas in cancer the evidence is against the intermediate formation of a hexosephosphate in the production of lactic acid from glucose.

Summary.

1. The aerobic and anaerobic glycolysis of cancer tissue is inhibited by pancreatic extract.

2. Other amylase-containing extracts made from malt diastase, taka-diastase and salivary gland also inhibit glycolysis in cancer tissue.

3. Cancer tissue is unable to form lactic acid from hexosediphosphate or hexosemonophosphate.

4. In the soluble muscle enzyme system, the production of lactic acid from hexosediphosphate is inhibited by pancreatic extract, while the production of lactic acid from hexosemonophosphate is not inhibited by pancreatic extract.

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